Synopsis of the thesis

PRODUCTION OF PHENOLICS, 1-DEOXYNOJIRIMYCIN AND RESVERATROL FROM *IN VITRO* CULTURE OF MULBERRY AND THEIR BIOLOGICAL ACTIVITY

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By

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Introduction

The wild edible plants have gained notable importance and applications in the food industry due to their promising role as functional food ingredients. These plants with their unique bioactives can replace the use of artificial compounds in food products (Donno et al., 2015). Many of the functional attributes of plants are mainly due to the presence of a major class of secondary metabolites i.e., phenolic acids and flavonoids (Kumar et al., 2020). The variation in nutritional, content of bioactives and nutraceutical potentials of the wild plants depends mainly on the growing condition, agronomic region of harvest and their origin (Soares et al., 2021). Many nutrition-rich under-exploited crops are suffering from a lack of attention from nutraceutical and economic perspectives (Berru et al., 2021). Exploring such crops for value addition could be a possible future source of income for the rural population (Soares et al., 2021). In recent years, the quest for industrially important crops with high nutritional value has increased the interest in the mulberry plant, which is an underutilized fruit crop (Krishna et al., 2018).

The genus *Morus,* commonly known as mulberry, belongs to the Moraceae family. It is a medicinal plant, which is a fast-growing deciduous tree native to the temperate, warm, and subtropical parts of Asia (Lee & Hwang, 2017). India is the second-largest mulberry growing country in the world and the main species like *M. alba*, *M. indica. M. serratea* and *M. laevigata* are grown in the northern part of the country (Krishna et al., 2018). However, most of the cultivated Indian genotypes belong to *M. indica.* Mulberry leaves form the major economic component in sericulture as they are valued for their foliage, which constitutes the main food for silkworms (Krishna et al., 2018). Similarly, the unripe and ripe fruits are traditionally used for the preparation of tonics, antidiabetics and immunostimulants (Jan et al., 2021). In European countries, the value-added products from mulberry are commercially sold and easily available, however in India, this versatile medicinal plant is still missing its identity at commercial and industrial scale. Mulberry is a rich source of high-value metabolites such as resveratrol, 1-deoxynojirimycin, anthocyanin and other polyphenolic

compound. They exhibit excellent anti-microbial, anti-hyperglycemic, anti-hyperlipidemic, anti-inflammatory, anti-cancer effects and is used to combat different acute and chronic diseases (Jan et al., 2021).

1-Deoxynojirimycin (DNJ), an iminosugar, is one of the most important biologically active natural compounds synthesized in mulberry leaves. It has structural similarity to the natural substrate (D-glucose) and is capable of binding to glycosidase and glucoamylase enzymes (Wulandari et al., 2019) thus reduces blood glucose levels, thereby preventing diabetes mellitus (Zhang et al., 2019). Likewise, anthocyanins, polyphenolic water-soluble pigments, is the largest group of polyphenolic pigment present in mulberry fruits. Anthocyanin-rich foods play a significant role in the prevention of various chronic diseases, including cardiovascular disease, type 2 diabetes, and some malignancies. Anthocyanin supplementation successfully decreases LDL and cholesterol (Wallace and Giusti, et al., 2019). The other high-value metabolite, resveratrol (trans–3, 4’, 5–trihydroxy stilbene) is a naturally occurring non-flavonoid stilbene. A wide variety of plants synthesize resveratrol in response to stress, injury, fungal infection, and ultraviolet radiation. Mulberry fruits, leaves, stems, and roots are major sources of resveratrol, which contributes to the biological activity of this plant (Shrikanta et al., 2015). Resveratrol is mainly known for protecting against coronary heart disease and preventing obesity (Buhroo et al., 2018).

In most mulberry-growing countries, especially India, their production is focused on enhancing the foliage for sericulture uses (Buhroo et al., 2018). Even though mulberry plants are a rich source of biologically important compounds, the quality and quantity of the phytochemicals collected from wild and field-grown plants are often fluctuating and heterogeneous depending upon the environmental conditions. The content of nutritional and functional components is significantly affected by the cultivar, harvesting time, season, and stage of maturity (Soares et al., 2021). Hence isolation of high-value secondary metabolites from the wild or cultivated plants or their chemical synthesis is not economically feasible. The biotechnological production specifically by plants tissue

culture to produce phytochemicals forms an alternative strategy to combat the conventional method which has great potential (Espinosa-Leal et al., 2018).

Plant tissue culture hold a great promise for the controlled production of myriad valuable secondary metabolites. The biomass and metabolite productivity of *in vitro* cultures are dependent on media composition, pH, inoculum density, culture environment like temperature, light density, agitation, aeration, etc. (Chandran et al., 2020). Thus, to improve the growth and metabolite productivity these factors must be optimized. The choice of suitable culture media and plant growth regulators plays a vital role in enhancing secondary metabolites production (Cardoso et al., 2019). Advances in scale-up approaches along with elicitation and precursor feeding techniques contribute to a significant increase in the number of applications of plant cell cultures to produce value-added metabolites. Cell suspension cultures represent an effective means for the bioreactor-based large-scale production of therapeutically relevant secondary metabolites. The latter offer advantages in terms of cost-effectiveness, large-scale production, and safety as compared with other production systems. Hence, plant biotechnology may supplement traditional agriculture on an industrial scale (Efferth et al., 2019).

Significant progress has been made in the field of plant tissue culture for the *in vitro* propagation, transgenic plant generation, somaclonal variation, development of polyploid and haploid mulberry, protoplast culture, somatic hybridization, and regeneration of whole plant (Sarkar et al., 2018). The literature reveals, despite the advances made in the biotechnological field to produce a wide range of value-added secondary metabolites from most of the medicinal plants, mulberry has been constrained mainly for micropropagation and regeneration of plants. A minor part of the available literature on mulberry highlights the production of resveratrol and DNJ in the *in vitro* culture of mulberry. However, no extensive quality evaluation on the phytochemicals of different cultivated varieties and the optimization of the medium for enhanced metabolite production has been carried out.

RESEARCH GAP

- No quality evaluation on the phytochemicals of different Indian cultivated varieties of mulberry and their suitability for special applications such as functional foods or nutraceuticals
- The fruits are under-exploited and lack the quality evaluation of bioactive compounds including polyphenols, DNJ and pigments
- Metabolite production from *in-vitro* culture of mulberry and the optimization of the medium for enhanced metabolite production has not been carried out
- No report demonstrating the large-scale production and the significant enhancement of the nutraceutically important secondary metabolites from mulberry

To the best of our knowledge, this is the first report demonstrating the large-scale production and the significant enhancement of the nutraceutically important secondary metabolites from mulberry. With this background knowledge, the present investigation was carried out with the following objectives.

Objectives

1. To screen phenolics, 1-deoxynojirimycin and resveratrol from mulberry varieties and evaluation of their biological activity.
2. To establish and optimize *in vitro* cultures for enhanced metabolite production.
3. To scale up key metabolites - 1-deoxynojirimycin and resveratrol.

Chapter 1. General introduction and review of the literature

This chapter gives brief information on mulberry plants, their habitat, and their use in traditional Ayurveda medicine. The economic viability of the mulberry leaves and their important role in the sericulture industry has been discussed. The review also provides information concerning the phytochemical constituents, functionality, nutraceutical potential of mulberry and the utilization of

fruits and leaf in the value-added products as a source of functional food. Subsequently, the problems or the drawbacks associated with the production of metabolites at commercial and industrial scale in from mulberry field-grown plants and the need for the biotechnological production of high-cost metabolites are included in the review. Furthermore, the potential of plant tissue culture for controlled production of valuable secondary metabolites has been listed. The review also provides a comprehensive discussion concerning the role of culture media composition, pH, inoculum density, culture environment like temperature, light density, agitation and aeration on the biomass and metabolite productivity of *in vitro* cultures. Advances in scale-up approaches along with elicitation and precursor feeding methods contribute to a significant increase in value-added metabolites is detailed.

Chapters 2, 3, and 4 deal with the committed objectives (1-3) of the current study. All the chapters include abstract, introduction, materials and methods, results and discussion, and conclusion of the objectives. Chapter 5 deals with a summary and conclusion. The cited references are mentioned at the end of the thesis.

Chapter 2. To screen phenolics, 1-deoxynojirimycin and resveratrol from mulberry varieties and evaluation of their biological activity.

The potential use of many native, easily available plant crops which are used for human consumption and value addition are not well recognized. Mulberry, being a traditional industrial crop rich in nutrients and nutraceuticals can be of great importance in the food industry. These plants are rich in unique metabolites and also are a good source of nutrition. However, mulberry leaves are mainly utilized in sericulture and are not exploited for their bioactive potential. Thus, the selection of promising mulberry cultivated variety, rich in bioactive compounds, like phenolic acid, resveratrol and 1-deoxynojirimycin (DNJ), increase their potential use as functional foods. The mulberry leaves from 9 different cultivated varieties Kanva-2 (K-2), Mysore Local (ML), Victory-1(V-1), S-36, S-13, Resource constraint-1 (RC-1), Resource constraint-2 (RC-2), G-2 and G-4 were

profiled for polyphenols. The data indicated that chlorogenic acid and kaempferol were the major polyphenols present in the nine selected cultivated varieties, in the range of 0.12-8.56, and 0.30-16.27 mg/100g fresh weight (FW), respectively. Protocatechuic acid, epicatechin and rutin were predominantly present in V-1, G-2, and ML (10.32, 7.96 and 12.14 mg/100g FW, respectively). Similarly, resveratrol and DNJ were highest in ML and K-2 (7.76 and 7.87 mg/100 g FW, respectively). Leaf extracts of G-2 and ML were able to effectively inhibit violacein production by 64.08% and 70.04%, respectively, at a concentration of 6 mg/mL, presumably due to a higher content of polyphenols. Chemometric evaluation of chromatographic data showed intraspecific variability and secondary metabolite co-existence in different varieties. Considering phytoconstituents, G-2, ML, K-2, and V-1 leaves could contribute efficiently to the rational utilization of mulberry in agro-food industries. Furthermore, G-2 and ML leaves can be a new source of quorum-sensing inhibitory agents.

Mulberry fruit is well recognized as one of the richest sources of bioactive compounds. We investigated the physicochemical composition and characterized the bioactive compounds during three stages of ripening in mulberry fruit. The proximate components, such as carbohydrates, proteins, and lipids, were found to be high in the ripe fruit compared to unripe and mid-ripe fruit. The ripe fruit contains higher total phenolics, flavonoids, and anthocyanin content (336.05, 282.55, and 220.87 mg/100g fresh weight (FW), respectively). The profiling of polyphenols showed that cyanidin-3-glucoside, epicatechin and resveratrol were the major polyphenols detected in the ripe fruit, with 155.31, 19.46, and 14.45 mg/100g FW, respectively. Chlorogenic acid and myricetin were predominant in the unripe and mid-ripe fruit (7.14 and 1.84 mg/100g FW, respectively). The highest content of DNJ was present in the mid-ripe fruit, with a content of 2.91 mg/100g FW. Thus, the results could help in the selection of appropriate developmental stages for food formulations rich in potential health components. Furthermore, plant bioactives have shown a strong anti-quorum sensing activity by effectively inhibiting violacein production. Thus, the mulberry fruit extracts

were subjected to anti-quorum sensing activity against *Chromobacterium violaceum*. Ripe fruit extracts showed the highest violacein inhibition with 76.30% at 1 mg/mL and, thus, could be used as a potent anti-quorum sensing source.

Conclusion

Mulberry leaves and fruits are used as a source of medicine and functional foods in many countries. In India, mulberry is considered an underutilized crop and the studies are restricted to the development of genotypes suitable for sericulture. However, the selection of promising mulberry variety and appropriate developmental stage of the fruit and their suitability for different applications could consequently promote its consumption in society. A significant difference in the phytoconstituent content was observed in mulberry leaves among different cultivated varieties and different ripening stages of the fruit. The results indicate that leaves from G-2, ML and V-1 were high in phenolic content and could be useful in improved revalorization of these underutilized leaves in the development of polyphenol-rich mulberry leaf-derived products. Similarly, the metabolites with potential health benefits like chlorogenic acid, DNJ and resveratrol were high in unripe, mid ripe and ripe fruits respectively. In addition, mulberry fruit and leaf extracts, especially ripe fruit extracts exhibited a potent anti-quorum sensing activity and are thus not only a rich source of nutrients and nutraceuticals but also a potent anti-QS agent. Furthermore, its commercialization can also offer employment and thus can improve the economy of the rural population.

Chapter 3. To establish and optimize *in vitro* cultures for enhanced metabolite production.

In the search for an alternative to produce medicinally important compounds from plants, biotechnological approaches, specifically plant tissue cultures, are found to have potential over conventional agriculture. Thus, this chapter deals with the establishment of *in vitro* cultures from different mulberry cultivated varieties, namely K2, ML, V1, S36, S13, RC1, RC2, G2 and G4 for screening of elite cell lines that produce higher content of polyphenols, resveratrol, and DNJ. Various combination of plant growth regulators was used for callus induction from leaves of

different mulberry cultivated varieties. The callus initiation was observed in the medium supplemented with 2,4-D (1 mg/L) and BAP (2 mg/L). The phenolic and flavonoid content were in the range of 123.51-384.19 and 10.87-70.60 mg/100 g respectively among different callus. Similarly, the HPLC analysis showed a high resveratrol content of 18.85 and 16.15 mg/100 g FW in V-1 and ML, respectively, and DNJ 5.05 and 4.27 mg/100g FW content were observed in K-2 and V-1 callus, respectively. Since V-1 showed the higher content of bioactive compounds, and the proliferation of callus was also at a higher rate compared to G-2, RC-1, RC-2, S-13 and S-36 and thus V-1 was selected for further analysis. Further, in order to enhance the metabolite content and biomass, the effect of light, sucrose, hormones, elicitors (methyl jasmonate-MJ, salicylic acid-SA), precursor feeding (phenylalanine-PA and tryptophan-TP) on the growth of callus and metabolite production were studied. The callus grown in the MS medium supplemented with 2,4-D (1 mg /L) + BAP (2 mg/L), 3% sucrose and incubated under diffused light showed higher metabolite content and further experiments were carried out using optimized culture conditions. Among the elicitors and percussors used at different concentrations to enhance the accumulation of metabolites, the highest TPC and TFC were recorded in the medium supplemented with TP2-0.5 mg/L and PA 200 µM respectively. Similarly, the highest resveratrol and DNJ accumulation were seen in the medium supplemented with TP2 and MJ 10 µM with a content of 55.84 and 24 mg/100 g FW on the 15^{th} and 25^{th} days, respectively. The selection of a high yielding variety along with the optimization of culture conditions could contribute to a significant increase in the number of applications of plant cell cultures to produce value-added metabolites.

Furthermore, the *in vitro* root cultures of mulberry were establishment to produce bioactive plant metabolites. Root initiation from the cultivated varieties was observed in the medium supplemented with different concentrations of 2,4-D and Kin. Best root was established in the V-1 leaves inoculated on MS with 2,4-D (1.5 mg/L) and Kin (0.3 mg/L). The total phenolic and flavonoid content were found to be 533.68 and 78.34 mg/100g FW, respectively. HPLC analysis

of polyphenols showed chlorogenic acid (11.13 mg/100g FW) as the major phenolics, and myricetin (8.75 mg/100g FW) and epicatechin (8.28 mg/100g FW) were the predominant flavonoids present in the *in vitro* roots of mulberry. Likewise, the resveratrol and DNJ content were found to be 2.16 and 2.91 mg/100g FW, respectively. The effect of phenylalanine precursor feeding with different concentrations on polyphenol and resveratrol accumulation was studied. The total phenolic content (662.46 mg/100g FW) was found to be highest in week 5, whereas the flavonoids (102.52 mg/100g FW) were high in week 6. HPLC analysis of resveratrol showed an increase in the content from 4.17 mg/100g FW to 9.55 mg/100g FW in the 5th week. These root cultures, on the other hand, provide a steady and reliable source of important metabolites in controlled environments, regardless of the weather or soil conditions.

Conclusion:

In the search for an alternative to produce medicinally important compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a complement to conventional agriculture in the industrial production. Their synthesis depends on biotic as well as abiotic factors like growth, temperature, light intensity, etc. The choice of suitable culture media plays a vital role in secondary metabolites production. The components of culture medium like macro and micronutrients, carbohydrates (sugars), and plant growth regulators like cytokinin and auxins influence the growth and metabolite production. Thus, various medium parameters and the culture conditions to improve the growth and metabolite productivity was optimized in the present study. The elicitors and precursor were studied to standardize the best condition for higher production of resveratrol and DNJ in a static culture of mulberry. The root culture showed lesser content of resveratrol and DNJ compared to the callus. The yield and the biomass were also relatively less significant, whereas the callus produced a significantly high content of polyphenols and resveratrol compared to the leaves from field-grown plants. Unlike traditional agriculture, plant tissue culture technology provides a continuous and reliable source of

important metabolites. The secondary metabolites-DNJ and resveratrol with pharmaceutical importance from the mulberry can be produced in controlled environments, independently from climate and soil conditions.

Chapter 4: To scale up key metabolites - 1-deoxynojirimycin and resveratrol.

The study aims at the establishment of suspension culture from mulberry callus by medium optimization and scaling up of resveratrol and DNJ production in a flask. The optimized culture media that resulted in the higher biomass and metabolite production in the V-1 callus were chosen for the establishment of submerged culture. Cell suspension culture is considered a potential method for the continuous production of commercially significant metabolites. To scale up the production of resveratrol and DNJ in a bioreactor, the medium condition was standardized at the flask level to give maximum production. Cell suspension cultures were established with different inoculum densities (4, 8, 12, and 16 g/L) in liquid medium supplemented with 30 g/L sucrose, 2,4-D (1 mg/L) and BAP (2 mg/L). Inoculum density with 12 g/L in the suspension medium showed higher biomass compared to other inoculums. Hence, the growth phase was studied at different rpms (80, 90, 110, and 120), and better cell growth was observed at 110 rpm. The time course of cell growth and metabolite production was recorded for up to 35 days, where samples were collected at an interval of 5 days. The highest biomass (54.51 g/L), phenolic acid (274.82 mg/L), and flavonoid (50.02 mg/L) content were observed on the 25th day of inoculation. The HPLC analysis of resveratrol (9.26 mg/L) and DNJ (2.61 mg/L) showed higher content on the 25th days of inoculation. To further enhance the metabolite production, the cells were treated with elicitors and precursors at the concentrations optimized in static culture, and the effects on biomass, total phenolic and flavonoid content, resveratrol, and DNJ augmentation were analyzed. TP showed maximum augmentation of resveratrol (19.25 mg/L), whereas the highest DNJ content (8.97 mg/L) was recorded in MJ. The treatments that resulted in higher levels of resveratrol and DNJ can be employed in the scale-up studies using a bioreactor.

To enhance the production of resveratrol and DNJ from mulberry culture flask levels to bioreactor (3.5 L) various studies were carried out. The optimized conditions for metabolite production at flask levels were scaled-up in a stirred tank bioreactor. Parameters such as pH, biomass growth, agitation, dissolved oxygen (DO), and production of resveratrol and DNJ were monitored for 30 days to characterize the kinetics of cell growth and metabolite production. Also, the growth kinetics were examined based on the biomass growth index along with metabolite production with supplementation of MJ, TP, and CO_2. Considering the effect of shear on biomass cells, low-shear axial type impellers were used with a linear increase in agitation from 100 to 200 rpm. Based on the growth phase, the increase in agitation favored the mass transfer kinetics. The impeller tip speed influences the mixing time and oxygen transfer as well as how cells are kept in suspension. By considering the process parameters for higher resveratrol production, the batch mode of operation was carried out with an initial biomass cell density of 12 g/L. Resveratrol production was higher with TP by 2.56 and 2.26-fold as compared to control and CO_2, respectively. Likewise, DNJ production was higher with MJ by 2.85 and 1.83-fold as compared to control and CO_2, respectively. The highest productivity of metabolites was recorded in MJ and the productivity of resveratrol and DNJ was enhanced from 0.52 to 1.54 and 0.31 to 0.76 mg/L/D, respectively in control and medium supplemented with MJ. However, the biomass growth of MJ and other treatments was significantly lower as compared with CO_2, implying an inverse relationship between biomass growth and metabolite production.

Conclusion:

Advances in scale-up methodologies and elicitation techniques contribute to a large growth in the number of plant cell culture applications for producing value-added compounds. Cell suspension culture, both on a flask scale and in a bioreactor, is being employed on a massive scale to cultivate plant cells from which secondary metabolites can be harvested. The primary purpose of aeration is to provide the biomass in suspension with sufficient oxygen for metabolic requirements, while

agitation should ensure that a uniform suspension of biomass cells is achieved in a homogenous nutrient medium. The agitator is required to achieve a few mixing objectives, such as bulk fluid and gas-phase mixing, air dispersion, oxygen transfer, heat, and mass transfer for uniform suspension in the bioreactor. Baffles are normally incorporated into agitated vessels to prevent a vortex and to improve aeration efficiency. Aeration may be enhanced using spargers in a bioreactor and hence, the orifice sparger was used in the present work. The above study confirms the stable production of polyphenols, resveratrol and DNJ in stirred tank bioreactor with the optimized culture conditions. With this, the application of bioreactors and their operation modes can be useful for the large-scale production of these high-value metabolites from mulberry with its varied health benefits and food applications.

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Significance of the work

- The understanding of the changes in metabolites among different varieties of leaf and ontogeny of fruits would help in identifying the appropriate raw material for development of functional foods.
- Unlike the traditional agriculture, plant tissue culture technology provides a continuous and reliable source of important metabolites.

- The secondary metabolites-DNJ and resveratrol with pharmaceutical importance from the mulberry can be produced in controlled environment, independently from climate and soil conditions.
- The present work could contribute to the effective utilization of biotechnological approach to produce nutraceutically important secondary metabolites with shorter harvest duration and aid in the conservation of natural population and sustainability.

Research outcome:

Shreelakshmi, S. V., Nazareth, M. S., Kumar, S. S., Giridhar, P., Prashanth, K., & Shetty, N. P. (2021a). Physicochemical composition and characterization of bioactive compounds of mulberry (*Morus indica* L.) fruit during ontogeny. *Plant foods for human nutrition, 76*(3), 304–310. https://doi.org/10.1007/s11130-021-00909-4

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